ON THE INTRACELLULAR SYNTHESIS OF COLLAGEN

by

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A b s t r a c t: Slices of experimental granulation tissue which had been incubated with ^{14}C-proline, other amino acids and glucose were homogenized, the subcellular particles fractionated with differential centrifugation and analyzed on labelled proline and hydroxyproline. The latter was found mainly in the incubation medium.

In another series ^{3}H-proline was allowed to incorporate similarly into the slices, which were homogenized and the particles fractionated by centrifugation in a density gradient. The postribosomal supernatant only contained labelled proline or hydroxyproline, proportionally to the protein content. The ^{3}H-hydroxyproline-containing components could not be identified.

The sedimentation pattern of the ribosomal fraction was determined by analytical ultracentrifugation. The differences in the patterns of granulomas of various ages were not considered conclusive.

The purpose of this work was to obtain information on (a) the presence of collagen precursors in subcellular fractions of the fibroblasts, and (b) the eventual changes in the ribosomal systems during the various phases in the development of the fibroblasts, *i.e.*, during proliferation, synthesis of collagen and involution. Preliminary work on the synthesis of collagen by homogenized granulation tissue had been unsuccessful. Therefore the synthesis of collagen had to be carried out in slices, which were subsequently homogenized and fractionated.

Corresponding studies on the synthesis of other proteins in the mammalian ribosomal system have been published, *e.g.*, by Campbell, Greengard & Kernot (1), Philipps (12), Manner & Gould (8) and on the synthesis of collagen by Kretsinger, Manner, Gould & Rich (6), who used chick embryos.

MATERIAL AND METHODS

Granulomas. — The experimental granulation tissue was produced essentially according to Viljanto (16). Pieces of moistened viscose-cellulose sponges (10 × 10 × 20 mm, dry weight 65—70 mg, from Säteri Oy, Ltd., Valkeakoski, Finland) were implanted subcutaneously on the backs of albino rats (weighing about 150 g, four pieces in each rat). The granulomas were dissected under ether anaesthesia, the capsules removed, and the granulomas immersed immediately into ice-cold 0.9 % (w/v) NaCl-solution. About 0.5 mm thick slices were cut in the cold room with Stadie-Riggs tissue slicer.

Experiments on subcellular fractions obtained with differential centrifugation. — Fifteen three-week granulomas (à 2 cm^3) were cut to slices, which were rinsed with cold Krebs-Ringer-phosphate solution. The slices were incubated for 4 hrs in 50 ml of Krebs-Ringer-phosphate, pH 7.4, which contained ^{14}C-proline (6 μc/50 ml; L-proline-^{14}C (U), specific activity 11.9 mc/mmole; The Radiochemical Centre, Amersham, Bucks., England) and the following amino acids in the indicated concentrations: L-leucine 0.76 mM, L-isoleucine 0.38 mM, L-arginine 0.95 mM, L-lysine 1.10 mM, L-tyrosine 0.22 mM, L-glutamine 0.85 mM and D-glucose 5.6 mM (3). In the first series the gas phase was air and in the second a mixture of oxygen (95 %) and carbon dioxide (5 %). After the incubation the slices were washed with cold Krebs-Ringer-phosphate solution containing in addition 0.01 % proline. The slices were homogenized with a Bühler-homogenizer (No. 21 00 00, E. Bühler, Tübingen, West Germany; nominal speed 50.000 rpm) into 0.25 M sucrose solution. An equal number of similar »carrier granulomas» were homogenized and added to the incubated material. In the second experiment the homogenate was treated additionally in Potter-Elvehjem apparatus (A. H. Thomas, Cat. size C; 4 times for 25 sec.).

The homogenate was first filtered through gauze. The nuclear fraction which also contained the cellular debris and tiny fibrils was obtained by centrifugation at 600 *g* for 15 mins in a refrigerated centrifuge and the sediment was washed once with 0.25 M sucrose solution. The supernatant was centrifuged at 5 000 *g* for 25 mins and the sediment which contained the mitochondria was washed once. The supernatant was separated to microsomal and cytoplasmic fractions by centrifugation at 105 000 *g* for 60 mins in a Spinco Model E centrifuge. The original incubation medium was cleared with a similar centrifugation and a small sediment was obtained.

For the analysis of radioactivity, the fractions were gelatinized in 10 ml of water at + 130° C for 3 hrs and the supernatants separated by centrifugation at 35 000 *g* for 15 mins. The sediments were washed once with boiling water. The combined supernatants were evaporated in a boiling water bath. The dry residues (= gelatinized collagen), sediments (= non-collagen proteins) and aliquots of the original subcellular fractions were hydrolyzed at + 130°C for 3 hrs in 5.7 N hydrochloric acid. The hydrolyzates were evaporated to dryness in a boiling water-bath, some water added and evaporation repeated twice. Finally the samples were decolorized with 0.5 g of activated charcoal.

The activities were determined according to Peterkofsky & Prockop (10) using a liquid scintillation equipment (NE 5503, Nuclear Enterprises Ltd., Medway, Sighthill, Edinburgh 11; high voltage 650 V and the input 50 mV).

Experiments on subcellular fractions obtained with centrifugation in a density gradient. — The slices were prepared from three-week granulomas, incubated for 2 hrs in Krebs-Ringer-phosphate, pH 7.4, as above. For four granulomas (à 2 cm^3) 10 ml of incubation medium was used containing 20 μc of ^{3}H-proline (L-proline-T(G); specific activity 224 mc/mmole; The Radiochemical Centre, Amersham, Bucks., England). Oxygen-carbon dioxide mixture was used as gas phase. After incubation the slices were rinsed five times with 60 ml of incubation fluid, blotted dry and frozen.

The slices were homogenized by forcing the deeply (—20°C) frozen mass through an aperture of 0.6 mm in diameter (equipment constructed in our workshop) and the homogenate centrifuged at 10 000 *g* for 20 mins in a refrigerated centrifuge. One ninth (v/v) of 5% Na-desoxycholate in 0.01 M tris-HCl buffer (pH 7.4, containing also 0.0015 M magnesium chloride and 0.01 M potassium chloride) was added to this postmitochondrial supernatant of the homogenate. A linear sucrose gradient (30—15%, volume 4 ml, was formed in a centrifuge tube ($\frac{1}{2} \times 2''$, volume 5 ml) and 0.4 ml of the postmitochondrial supernatant was applied above it. The gradient centrifugation was performed according to Kretsinger *et al.* (6) at 29 500 rpm. for 120 mins in the SW-39E rotor in a Spinco Model E centrifuge. The contents of the tubes were aspired from the bottom and divided to 3-drop samples — about 30 from each centrifuge tube.

To each 3-drop sample 2 ml of water was added and the light absorption at 260 mμ was measured with a Beckman Model DU or DB spectrophotometer. The protein content was measured from 0.5 ml aliquots (7).

The samples were hydrolyzed at + 130°C for 3 hrs in 5.7 N hydrochloric acid, the hydrolyzates evaporated in a water bath and the evaporations repeated twice. The dry residues were dissolved into 8 ml of water, and 1 ml of carrier solution was added, containing 1 mg of hydroxyproline and 10 mg of proline. Finally the samples were treated with 100 mg of activated charcoal. The pellets in the gradient tubes were washed with tris-HCl buffer at 160 000 *g* for 60 mins, hydrolyzed and analyzed like the other fractions. The activities of ^{3}H-proline and ^{3}H-hydroxyproline were measured according to Peterkofsky & Prockop (10) using a Packard Liquid Scintillation Spectrometer (Model 3214).

The proteins in the 8 top fractions which contained most of the radioactivity (Fig. 1) were studied from a combined sample. To identify the eventual collagen components, supernatant was mixed with acid-soluble rat-tail tendon collagen, denatured for 15 mins at +40°C and fractionated with starch gel electrophoresis (9). For the demonstration of the non-collagenous proteins the supernatant was fractionated in starch gel electrophoresis according to Poulik (13). In both cases the resulting gel sheets were cut perpendicularly to 1 mm strips, which were treated for radioactivity measurements as described above in regard to the gradient fractions.

The supernatant in the combined 8 top fractions was also dialyzed against 0.9% NaCl for 2 days at +4°C and the radioactivities in the retentate were measured.

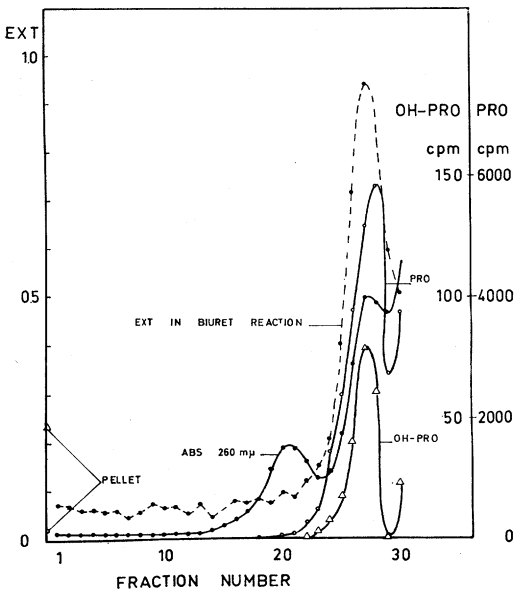

Fig. 1. — Distribution of protein, UV-absorbing material, ^{3}H-proline and ^{3}H-hydroxyproline activities in the density gradient fractions. Sedimentation from right to left.

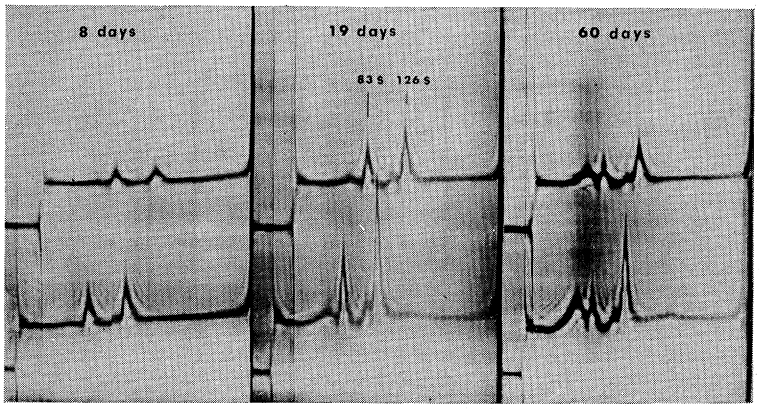

Fig. 2. — Ribosomal sedimentation patterns from granulomas of indicated ages. The figures are taken 8 mins after reaching the final speed, 42 040 rpm. Phase plate angle 50°.

Sedimentation analysis on the ribosomal fraction from granulomas of various ages. — The granulomas were 7—8 days, 19—22 days and 60 days of age. Ten granulomas in each group were homogenized into 25 ml of 0.03 M tris-HCl buffer, pH 8.2, containing 5 mM of magnesium chloride and 0.25 M of sucrose, first with the above-mentioned water-cooled Bühler-homogenizer (4×30 sec. with 90 sec. intervals). The homogenization was completed with the described Potter-Elvehjem apparatus, immersed into ice-water, applying four strokes of the plunger to the bottom.

The debris, nuclei and mitochondria were removed with centrifugation at 17 000 *g* for 10 mins in a refrigerated centrifuge. To this post-mitochondrial supernatant one ninth (v/v) of 5% Na-desoxycholate in tris-HCl buffer was added drop by drop, while stirring. The centrifugation for the removal of the mitochondria was repeated. The ribosomes were sedimented by centrifugation at 105 000 *g* for 60 mins at 0—+4°C in a Spinco Model E centrifuge. The combined ribosomal sediments were suspended in 30 ml of 0.03 M tris-HCl buffer, pH 7.5, containing 5 mM of magnesium chloride, where the ribosomes are supposed to be stable and homogenized with the Potter-Elvehjem's homogenizer (four strokes, about 15 sec. each). The centrifugations for the removal of the mitochondria and subsequently for the sedimentation of the ribosomes were repeated once, and the purified ribosomes resuspended into 2 ml of the tris-HCl buffer and homogenized again. This ribosomal suspension was finally centrifuged at 18 400 rpm. (23 400 *g*) for 15 mins in a refrigerated centrifuge.

From this final ribosomal supernatant analytical ultracentrifugations were run according to Petermann & Pavlovec (11) in two concentrations at +2.5—+5°C (Fig. 2). The rotor D of a Spinco Model E ultracentrifuge was used at 42 040 rpm. The sedimentation coefficients were corrected to water at +20°C applying $\overline{v} = 0.664$ ml/g (4) and extrapolated linearly to zero concentration. The molecular weights of the ribosomes were estimated from the empirical formula (5):

$$s^0_{20,w} = 0.43 \times 10^{-2} \times M^{0.65}$$

RESULTS AND DISCUSSION

Incorporation of proline in the subcellular fractions obtained with differential centrifugation. — The data have been collected in Table 1. In the actual subcellular fractions only the nuclear and the cytoplasmic fractions contained labelled hydroxyproline. The nuclear fraction also contains the fine fibrils and the ratio of hydroxyproline/proline activities is high. The microsomal fraction is almost devoid of any activity and intermediates of collagen synthesis produced in the microsomes may be attached to them for a short period only or are detached to the cytoplasm during the homogenization.

The surprising finding is the high activity in the incubation medium both in sedimentable and soluble form. It may be suspected that the activity of ^{14}C-hydroxyproline cannot be estimated accurately in the presence of such a high excess of ^{14}C-proline, but the order of magnitude should be right. We did not study whether the ^{14}C-hydroxyproline was in the form of small peptides or in some soluble form of collagen.

Incorporation of proline in the subcellular fractions obtained with gradient centrifugation. — In no case was ^{3}H-labelled hydroxyproline observed in the particle fractions, but only in the postribosomal supernatant. The activities of proline and hydroxyproline correlated with the concentration of total protein. All the hydroxyproline was non-dialyzable but from the labelled proline only one sixth remained inside the bag. No labelled conventional components of collagen could be demonstrated. Five non-collagenous bands were detected in starch gel

TABLE 1

INCORPORATION OF ^{14}C-PROLINE INTO VARIOUS FRACTIONS OF INCUBATED GRANULOMA SLICES

Fraction / Exp. No.	^{14}C-Proline				^{14}C-Hydroxyproline			
	Total Protein		Collagen	Non-Collagen	Total Protein		Collagen	Non-Collagen
	1	2[1]	2	2	1	2[1]	2	2
Actual subcellular fractions:								
Mitochondrial	530	1 600	310	1 300	40	20	20	0
Microsomal	3 200	18 700	1 100	17 500	50	70	30	40
Cytoplasmic	3 800	27 600	8 300	19 200	130	380	380	0
Total	7 530	47 900	9 710	38 000	220	470	430	40
Nuclei, debris and small fibrils	4 900	10 000	3 600	6 500	290	810	640	170
Incubation fluid:								
supernatant	417 000	345 000	333 000	12 000	19 000	5 700	5 700	0
sediment	12 500	22 200	14 500	7 800	960	1 200	1 200	0
Total	429 500	367 200	347 500	19 800	19 960	6 900	6 900	0

[1] Obtained by addition. The activities are expressed as cpm.

electrophoresis according to Poulik (13), but none of them contained detectable radioactivity. The pellet contained relatively large amounts of active proline and hydroxyproline.

It is apparent that the polysome system is very easily degraded during the manipulations. This may be the reason why the synthesis of collagen cannot be demonstrated with homogenized granulation tissue. It remains to be studied whether the hydroxyproline-containing material in the supernatant fraction originates from broken polysomes.

Ribosomal patterns in granulomas of various ages. — The main components in the patterns (Fig. 2) were the two sharp peaks sedimenting with velocities of 83 S and 126 S. Between them a small peak (111 S) was observed and in some runs there were indications of larger aggregates (150—200 S.) Relying on the empirical formula of Inouye *et al.* (5) the 83-S material represents the monomeric and the 126-S material the dimeric ribosomes, with molecular weights of 3.9 and 7.5 millions respectively. The component which sediments with 66 S seems not to be of ribosomal origin but rather a metalloprotein (15).

No conclusive qualitative differences could be observed between the various developmental phases of the granulation tissue. This result does not exclude the presence of large aggregates which may be broken down by the rather vigorous homogenization which is necessary to break the cells inside the mechanically strong sponge. Therefore the quantitative differences between the various ages have to be considered with reservations.

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